Exhibit 3

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A liposome-PCR assay for the ultrasensitive detection of biological toxins

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We describe an ultrasensitive immunoassay for detecting biotoxins that uses liposomes with encapsulated DNA reporters, and ganglioside receptors embedded in the bilayer, as a detection reagent. After immobilization of the target biotoxin by a capture antibody and co-binding of the detection reagent, the liposomes are ruptured to release the reporters, which are quantified by real-time PCR. Assays for cholera and botulinum toxins are several orders of magnitude more sensitive than current detection methods.

The potential use of biological toxins as weapons of mass destruction has created an urgent need for rapid and highly sensitive assays for their detection. We describe one such assay method, liposome polymerase chain reaction (LPCR), which is robust and straightforward to perform, yet uses inexpensive and stable reagents.

We present an overview of the LPCR method with an assay for the detection of cholera toxin beta subunit (CTBS) in deionized water. Detailed procedures for this assay and one for botulinum neurotoxin type A (BoNT/A) are provided in the Supplementary Methods online. About 60 copies of an ~80-bp dsDNA segment (the reporter) are encapsulated inside a single-shell liposome. The reporter serves as a PCR amplification substrate for quantification of CTBS. Approximately 2,500 molecules of monosialoganglioside G_{M1} are incorporated into the bilayer of the liposome to serve as a nonspecific receptor for CTBS[1]. A cross-section of the resulting liposome detection reagent is represented in Figure 1.

The LPCR assay follows the familiar sandwich enzyme-linked immunosorbent assay (ELISA) format. A monoclonal antibody against CTBS is adsorbed inside the wells of a microtiter plate and serves to provide specificity by capturing CTBS from the sample solution. Nonspecific protein binding is blocked using bovine serum albumin. Each well then receives 150 µl of serially diluted CTBS (a concentration range of 10^{-14} to 10^{-19} M) or buffer (blank). Nonspecific liposome binding is blocked by the addition of small unilamellar vesicles composed of phosphatidylcholine. The liposome detection reagent is added, and the plate is incubated at 23 °C for 1 h. The plate wells are then rinsed several times with PBS. Unencapsulated DNA is degraded by the addition of pancreatic DNase I solution, followed by incubation at 37 °C for 30 min. The DNase I is then inactivated by heating the plate to 80 °C for 10 min, and the encapsulated reporters are released by rupturing the liposomes with Triton X-100. An aliquot from each microtiter plate well is added to a PCR reaction mixture, and the samples are analyzed by real-time PCR (Supplementary Fig. 1 online).

Figure 2a shows the average cycle threshold (Ct) values obtained by real-time PCR versus the log of the number of CTBS molecules per plate well for four replicate LPCR measurements of CTBS in deionized water. The detection threshold of the assay is defined as the average Ct value of the blank minus three times the standard deviation of the

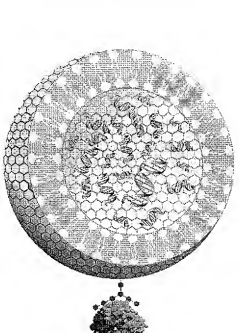

Figure 1 Representation of a liposome detection reagent in cross section. The dsDNA reporters (green with red bars) are encapsulated inside the bilayer (yellow) into which a monosialoganglioside G_{M1} receptor (blue) has been incorporated. The liposome is shown bound to a CTBS pentamer, which is co-bound to a capture antibody.

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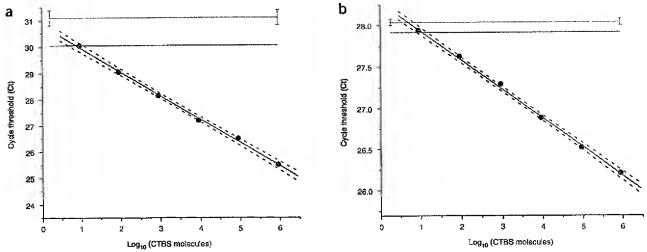

Figure 2 Plots of the average serial dilution Ct values versus the log of the number of molecules per plate well for the four replicate measurements of (a) CTBS in deionized water and (b) BoNT/A in deionized water. The concentration ranges for both CTBS and BoNT/A were 10^{-14} to 10^{-19} M. Solid black circles are average Ct values. The solid red line is a linear regression fit ($r^2 = 0.999$) to the average Ct values, and the dashed blue lines are the upper and lower 95% confidence limits. The solid horizontal orange line denotes the average blank Ct value, with the standard deviation of the blank drawn at each end of this line. The solid horizontal green line intersecting the linear regression line indicates the detection threshold of the LPCR assay as defined in the text.

blank, as is used for immuno-PCR assays[2]. The detection threshold for this assay is 10 ± 3 molecules of CTBS (0.02 fg/ml) based upon the linear regression and 95% confidence limits derived from the sample data. The dynamic range of the assay is almost five orders of magnitude. The LPCR dose-response curve is similar in linearity and dynamic range to those seen with conventional immuno-PCR[2,3], but with higher sensitivity. The specificity of the assay for CTBS was demonstrated in an LPCR assay in which tetanus toxoid was substituted for CTBS; this assay revealed no detectable reporter amplification above background levels (not shown).

LPCR assays were also performed on a specimen of field run-off water collected from a local farm and a specimen of human urine, both spiked with CTBS (**Supplementary Figs. 2** and **3** online). The detection threshold for the water specimen is 377 ± 168 molecules of CTBS (0.75 fg/ml). The detection threshold for the urine specimen is 43 ± 10 molecules of CTBS (0.09 fg/ml).

To detect BoNT/A in deionized water, we used a commercially available affinity-purified polyclonal rabbit IgG antibody against this biotoxin. The liposome detection reagent was prepared as described above, but with 2 mol% trisialoganglioside G_{T1b} in place of monosialoganglioside G_{M1}. Trisialoganglioside G_{T1b} is a component of the binding site for the carboxy-terminal half of the 100-kDa heavy chain of BoNT/A[4]. Other minor changes relative to the assay for CTBS are described in the **Supplementary Methods**. The results of this assay are shown in **Figure 2b**. The detection threshold is 12 ± 4 molecules (0.02 fg/ml). The assay is linear over approximately five orders of magnitude.

The sensitivities of the LPCR assays for CTBS and BoNT/A are compared with those of other biotoxin assay methods in **Supplementary Table 1** online. The LPCR detection thresholds for CTBS and BoNT/A are 2–3 orders of magnitude lower than those reported by the most sensitive assays currently in use, while maintaining high specificity and having assay times equal to or shorter than those of most biotoxin assays.

LPCR offers several advantages over current biotoxin detection methods. First, derivatization of the reporter or ganglioside receptors is not required. The reporter is freely encapsulated inside the liposomes, and the ganglioside receptors spontaneously partition into the bilayer as the liposomes are formed. This greatly simplifies the preparation and purification of the detection reagent. Second, the use of real-time PCR, rather than end point PCR, improves the quantitative accuracy of the assay; it also allows for improved precision by performing replicate measurements on the samples and applying statistical treatment to the data[5]. Third, LPCR displays 100–1,000 times greater sensitivity than previous assays for biological toxins (see **Supplementary Table 1**). This is due, in part, to the high number of reporters per binding event and the low nonspecific binding of the liposome detection reagents. Fourth, sequestration of the reporters inside the liposomes offers two distinct advantages not possible with other immuno-PCR-based assays. The reporters are protected from chemical or enzymatic degradation by impurities present in the sample that are incompletely removed during the wash steps. This substantially reduces the possibility of false-negative results. The more important advantage of encapsulating the reporters is that DNase I can be used to degrade any contaminating DNA present in the microtiter plate wells immediately before the rupture of the liposomes by detergent. Thus, DNA contamination from the assay environment, from incomplete purification of the liposome detection reagents, from carryover by pipette tips or plate washer nozzles, or genomic DNA contamination remaining from the samples can all be eliminated. This substantially reduces the possibility of false-positive results and improves the sensitivity and precision of the assay.

The application of LPCR could be greatly expanded by coupling antibodies to the liposome surface as receptors in place of gangliosides. Phospholipid anchors for antibodies are commercially available, and they will spontaneously partition into the bilayer. A number of straightforward coupling chemistries exist for linking whole antibodies, or Fab′ fragments, to the phospholipid anchors[6]. In addition, the

encapsulated dsDNA reporter can be sequence coded to the antibody covalently linked to the liposome detection reagent, which would allow simultaneous detection of multiple antigens. We are currently using this approach for the detection of additional chemical and biological warfare agents and for the detection of biomarkers for cancer and other diseases.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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